PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Markus HECKER & Andreas H. WAGNER	Group Art Unit: 1632
Serial No.: 10/526,430	Examiner: D. Montanari
Filed: March 1, 2005	Atty. Dkt. No.: DEBE:052US/SLH
For: PHARMACEUTICAL FORMULATION WITH NONSTEROIDAL ANTIPHLOGISTICS AND NUCLEIC ACIDS FOR TRANSFERRING NUCLEIC ACIDS INTO EUKARYOTIC CELLS	Confirmation No.: 9671

CERTIFICATE OF ELECTRONIC SUBMISSION

DATE OF SUBMISSION: October 16, 2008

DECLARATION OF GERHARD BURCKHARDT UNDER 37 C.F.R. §1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

I, Prof. Dr. Gerhard Burckhardt, do declare that:

1. I am a citizen of Germany residing at Göttingen. I currently hold the position of Full Professor and Chairman at the Institute of Physiology of the Georg-August-University Göttingen. My research experience includes well over 120 original articles in peer reviewed international scientific journals and close to 22 review articles in scientific

journals, journal supplements, conference proceedings and books. I have been trained in Medicine and hold university degrees including a doctorate with distinction in Medicine (Dr. med.). I have worked in basic research for almost 35 years, mainly focusing on transport of organic anions across cell membranes. I have a special expertise in renal excretion mechanisms for anionic drugs. A copy of my *curriculum vitae* is attached.

2. Optimization of the pH of a solution containing decoy oligonucleotides (dODNs) or DNA is critical for the delivery of these compounds to the interior of the target cell. Presently, it is not known at a molecular level how dODNs and DNA cross cellular membranes. A simple diffusion can be firmly excluded because of the high water solubility that is due to the presence of numerous negative charges in these molecules. Therefore, it remains that dODNs and DNA are taken up into cells by anion channels, a transporter of the solute carrier family (SLC family), and/or by endocytosis.

3. Anion channels are – in my opinion – unlikely candidates for dODN or DNA uptake. Given the inside negative membrane potential in the range of -40 to -70 mV, these channels would facilitate the efflux of the highly negatively charged dODN or DNA rather than providing an entry pathway against the membrane potential.

4. Among the transporters, SLC19A1, the reduced folate carrier-1 (RFC1 [1]) is a strong candidate for dODN or DNA uptake because this uptake can be competed out by folate. This transporter that is expressed in virtually all tissues of the human body, has a relatively low affinity for folate, and a high affinity for the antineoplastic methotrexate. It

is believed that folate uptake by RFC1 occurs together with the uptake of protons (or the release of hydroxyl ions). An acidification of the extracellular solution (= increase of proton concentration or decrease of hydroxyl ion concentration) would enhance the activity of RFC1. The adjustment of pH is, therefore, critical for the function of RFC1, and would similarly be important to RFC1-mediated uptake of dODN or DNA. Moreover, the pH dependence of RFC may differ between cells / tissues and depend on the phosphorylation status of the transporter [2]. It appears that in many cells the optimal folate transport activity by RFC1 is at pH 7.4, which is higher than observed by the present inventors in their studies. This would argue against a major contribution of RFC1 to the uptake of dODNs and DNA at low pH, but does not exclude a major involvement of RFC1 in uptake at physiological pH (7.4).

5. Besides RFC1, folate receptors (FR) may be responsible for dODN and DNA transport. FR may be involved in folate delivery to target cells [3,4]. These receptors bind folate with very high affinity and are endocytosed together with folate ("cargo"). After acidification of the endosomes and dissociation of receptors and folate, the cargo is released into the cytosol, possibly by anion transporters, and the FR is recycled back to the membrane. Binding of folate to its receptor is enhanced at acidic pH, and so is delivery of folate into RF-expressing cells [5]. Again, adjusting the pH of the solution is critical for the activity of the uptake process through FR. In addition, the release of folate from endosomes into the cytosol is pH-dependent, being faster at acidic pH.

6. It is known that a number of molecules can be taken up through the FR, if coupled to folate. If, as predicted, dODNs or DNA are taken up by FR-induced endocytosis, an acidic pH will enhance uptake, as was in fact observed by the present inventors. Moreover, it is possible that the pH dependence is different for different cargo molecules (dODN; DNA) and, thus, needs to be adjusted individually dependent on the physicochemical properties of the cargo.

7. In conclusion, results of the present inventors suggest that, at a pH of 6.4, dODNs and DNA are preferentially taken up into target cells by binding to the folate receptor and subsequent endocytosis. At a physiological pH of 7.4, uptake of dODNs and DNA may occur mainly through RFC1. Besides pH, structural differences of dODNs and DNA will influence the affinity for binding and translocation by both mechanisms and impact on the relative contribution of RFC1 and FR to overall uptake. The adjustment of pH therefore involves elaborate studies on the uptake of each individual dODN or DNA into model cells as a function of extracellular pH. If uptake cannot be measured directly, e.g. due to the lack of radiolabeled probes, the pH dependence of the cellular effects elicited by dODNs or DNA can serve as a readout. While further experiments will clarify, at a molecular level, the specifics of the transport system for dODNs and DNA, it is already clear that the adjustment of pH in this system is neither trivial nor mere optimization of a routine aspect.

8. I declare that all statements made herein of my own knowledge are true, and that all statements of my own belief are believed to be true, and further that these statements

were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of title 18 of the United States Code.

October 16, 2008
Date

Prof. Dr. Gerhard Burckhardt

Cited Literature:

[1] Ganapathy V, Smith SB, Prasad PD. Pflügers Arch – Eur J Physiol 447:641-646, 2004.

[2] Rajgopal A, Sierra EE, Zhao R, Goldman ID. Am J Physiol Cell Physiol 281:C1579-C1586, 2001.

[3] Anthony AC. Blood 79:2807-2820, 1992.

[4] Leamon CP, Low PS. Drug Discov Today 6:44-51, 2001.

[5] Sierra EE, Brigle KE, Spinelly MJ, Goldman ID. Biochem Pharamcol 50:1287-1294, 1995.

Curriculum vitae

Name	Gerhard Burckhardt
Date and Place of Birth	August 1, 1947, Kaiserslautern (Germany)
Nationality	German
Marrital Status	Married with PD Dr. Birgitta Christina Burckhardt Daughter: Friederike Sofie Burckhardt, born June 16, 1982
Academic Education	Medicine, Johann Wolfgang Goethe-Universität, Frankfurt am Main, 1966-1972 *Staatsexamen* (Final Examination) August 9, 1972 ("Very Good")
Promotion	*Dr. med.*, March 29, 1974 Title of Thesis: Determination of K^+ Transport Sites in Ehrlich Mouse Ascites Tumor Cells ("summa cum laude")
Habilitation	Habilitation for *Physiology*, February 4, 1988 Title of Habilitation Thesis: Na^+-H^+ exchange and ATP-driven H^+ Transport in Proximal Tubules of Mammalian Kidney *Privatdozent* (Permission to Teach), May 19, 1988
Professional Career	*Studiendekan* (Subdean for Student Affairs) since April 2008 *Universitätsprofessor* und Direktor (Full Professor and Chair) of the Department Vegetative Physiologie und Pathophysiologie, Georg-August-Universität Göttingen, September 1991 - *Wissenschaftlicher Angestellter* (Research Assistant) at the Max-Planck-Institut für Biophysik, Frankfurt am Main; Department of Physiology (Director: Prof. Dr. med. K. J. Ullrich), September 1978 to August 1991 *Wissenschaftlicher Angestellter* at the Gustav-Embden-Zentrum der Biologischen Chemie (Department of Biochemistry), September 1973 to August 1978 *Medizinalassistent* (Internship), September 1972 to August 1973 *Approbation als Arzt* (Licence) September 3, 1973
Awards, Grants, special functions	Spokesman and Director of the Graduiertenkolleg (PhD Program) 335 "Clinical, Cellular, and Molecular Biology of Internal Organs", 1997 to 2006 President of the German Physiological Society, 2005 Member of the Deutsche Akademie der Naturforscher Leopoldina since 2002 Member of the SFB (Collaborative Research Center) 402 "Molekulare und zelluläre Hepatogastroenterologie" (Spokesman Prof. Dr. K. Jungermann), Göttingen, 1994 to 2000 Member of the SFB 169 "Struktur und Funktion membranständiger Proteine (Spokesman: Prof. Dr. Dr. H. Fasold), Frankfurt, 1984 to 1991 *Preis des Jahres 1975 zur Förderung des Wissenschaftlichen Nachwuchses* donated by the Paul-Ehrlich-Stiftung (Award for the Doctoral Thesis, 1975)
Organisation of Meetings	84th Annual Meeting of the German Physiological Society, Göttingen, March 2005 (Organizer) Transport Colloquium Rauischholzhausen, Marburg, 1999, 2001, 2003, 2005 (Co-organizer) Göttinger Transporttage since 1999 (annually; Organizer) Membranforum "Identifizierung und Molekulare Charakterisierung von Transportproteinen"; Frankfurt, 1987 (Co-organizer) International Symposium "Epithelial Anion Transport - Hormonal Regulation", Frankfurt/Main, 1985 (Co-organizer)

Göttingen, October 14, 2008 G. Burckhardt